Protection of Humans against Malaria by Immunization with Radiation-Attenuated *Plasmodium falciparum* Sporozoites

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During 1989–1999, 11 volunteers were immunized by the bites of 1001–2927 irradiated mosquitoes harboring infectious sporozoites of *Plasmodium falciparum (Pf)* strain NF54 or clone 3D7/NF54. Ten volunteers were first challenged by the bites of *Pf*-infected mosquitoes 2–9 weeks after the last immunization, and all were protected. A volunteer challenged 10 weeks after the last immunization was not protected. Five previously protected volunteers were rechallenged 23–42 weeks after a secondary immunization, and 4 were protected. Two volunteers were protected when rechallenged with a heterologous *Pf* strain (7G8). In total, there was protection in 24 of 26 challenges. These results expand published findings demonstrating that immunization by exposure to thousands of mosquitoes carrying radiation-attenuated *Pf* sporozoites is safe and well tolerated and elicits strain-transcendent protective immunity that persists for at least 42 weeks.

In 1967, Nussenzweig et al. [1] reported that immunizing mice with radiation-attenuated *Plasmodium berghei* sporozoites protected them against challenge with fully infectious sporozoites. These rodent studies provided the impetus for human studies, and, during the 1970s, Clyde and colleagues [2–5], Rieckmann and colleagues [6–8], and McCarthy and Clyde [9] established that immunizing human volunteers with the bites of irradiated mosquitoes carrying *P. falciparum (Pf)* or *P. vivax (Pv)* sporozoites in their salivary glands could protect humans against challenge with fully infectious *Pf* or *Pv* sporozoites. These studies demonstrated that a malaria vaccine offering sterile protective immunity was possible. However, it has been considered to be clinically and logistically impractical to immunize large numbers of susceptible persons with the irradiated sporozoite vaccine, because the sporozoites must be delivered alive either by the bite of infected mosquitoes or, potentially, by intravenous injection, as is done with mice.

Therefore, many scientists have focused on understanding the clinical requirements for this protective immunity, the immune mechanisms responsible for the protection, and the antigenic targets of these protective immune responses and on developing vaccine delivery systems that induce such protection. Much of this basic work, carried out in *P. berghei* and *P. yoelii* rodent model systems, has yielded important insights into irradiated sporozoite vaccine–induced protection and has led to the development of a number of candidate vaccines (reviewed in [10–12]). In fact, the results from rodent and human model systems are strikingly concordant.

To better delineate the clinical characteristics and requirements for protecting humans with the irradiated sporozoite vaccine, to assess the protective immune responses elicited in humans, and to identify the antigens and epitopes on the proteins that elicited immune responses in humans, in 1989 we began immunizing volunteers with gamma radiation–attenuated *Pf* sporozoites. Preliminary clinical results and extensive immunologic assay results from these studies have been published [13–19]. These immunologic studies, combined with those of others [20–26], have increased our understanding of the immunologic responses in humans immunized with radiation-attenuated *Pf*

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sporozoites. Here we report the results of 10 years' clinical experience with these immunizations and challenges and compare our results with those of published clinical reports of human immunization with irradiated *Plasmodium* sporozoites.

Methods

Preparation of sporozoite-infected mosquitoes. The *Pf* asexual and sexual erythrocytic-stage parasites were grown in normal human erythrocytes, using standard culture medium containing 10% heat-inactivated (56°C, 30 min) normal human serum. All erythrocytes and sera were obtained from donors who were at low risk for both hepatitis and human immunodeficiency virus (HIV) infection and whose serum did not contain hepatitis B surface antigen or antibodies to hepatitis C, *Treponema pallidum,* or HIV. Blood and serum for culture were purchased (Interstate Blood Bank), and each shipment carried a certificate of analysis certifying that the blood products were negative or nonreactive for the above pathogens.

The mosquito species was *Anopheles stephensi*. Colonies of *A. stephensi* have been maintained at the Biomedical Research Institute/Naval Medical Research Center (NMRC) and the Walter Reed Army Institute for Research (WRAIR) for several decades. To date, no adventitious agent of disease has been detected within these mosquito colonies.

About 16–20 days before a volunteer immunization, female mosquitoes from a secure insectary were infected with gametocytes of the *Pf* NF54 strain or the 3D7 clone of NF54 [27], as described elsewhere [13, 28–31]. One hour before immunization, the female mosquitoes were exposed to 15,000 rad of gamma radiation from a ^{60}Co or ^{137}Cs source. This radiation dose is believed to be sufficient to attenuate the sporozoites, allowing them to enter hepatic cells and undergo partial development but preventing them from developing into mature liver-stage schizonts, and thereby eliminating their ability to infect erythrocytes [2–5, 13–15].

Study subjects. Healthy adult male volunteers aged 18–50 years were recruited from the US Navy, Army, and Public Health Service. Volunteers were enrolled if they met previously described eligibility criteria [13]. Due to the long-term duration of this study, approval was obtained from the institutional review boards before immunizing or rechallenging volunteers who passed age 50 years in the latter part of the study. The longest duration of participation was 10 years for 2 volunteers. Volunteer identification numbers, listed in table 1, are not consecutive, because the numbers were sequentially assigned at screening, and some potential volunteers did not meet eligibility requirements and were not enrolled.

Immunization of volunteers. For the purposes of this study, we defined 2 categories of immunizations: a primary immunization series and secondary immunizations. Primary immunizations were defined as occurring before the first challenge. Secondary immunizations were those after the first challenge. Sporozoites were administered to volunteers via the bites of hundreds of irradiated female anopheline mosquitoes, by a process described earlier [13]. Of the mosquitoes taking a blood meal, 50 were dissected to estimate the percentage of mosquitoes having a sporozoite gland score $\geqslant 2$ (usually 50%–75%, as described elsewhere [28]). This percentage was multiplied by the total number of mosquitoes taking a blood meal, to calculate the number of immunizing bites. A physician with emergency medical drugs and equipment was present during the immunization. Volunteers were observed for 30 min after each immunization for immediate adverse reactions to the mosquito bites.

Over the 10 years of our studies, volunteers were exposed to the bites of irradiated mosquitoes on 165 occasions, and no breakthrough blood-stage infections were detected. After immunization, blood smears were not routinely done. Volunteers were instructed to contact study physicians should fever (oral temperature $\geqslant 38°C$), headache, chill, myalgia, or malaise develop at any time within 6–28 days after an immunization session—allowing timely diagnosis and treatment without risking the onset of severe disease.

Immunizations were done no more than once every 2 weeks. The goal for the primary immunization series (i.e., all bites occurring before first challenge) was to immunize volunteers with >1000 bites from irradiated mosquitoes having a sporozoite gland score $\geqslant 2$ before their first challenge. However, 1 of the early volunteers in the study was first challenged after only 606 immunizing bites. Volunteers averaged 9 immunizations with a mean of 1071 immunizing mosquito bites per volunteer over 9–10 months before their first challenge. After the initial challenge, subsequent secondary immunizations with additional batches of irradiated mosquitoes were done to maintain or regain sterile protective immunity.

Challenge of immunized volunteers. Immunized volunteers were challenged with infectious mosquitoes to ascertain the development of protective immunity, since there were no surrogate markers for protection. The challenge method has been described elsewhere [28–30]. Volunteers were closely monitored by study physicians and were instructed to report fever (oral temperature $\geqslant 38°C$), headache, chill, myalgia, malaise, or other medical concerns. All volunteers were monitored daily with thick blood smears, beginning 7 days after challenge and continuing until day 28, and then were monitored weekly for 4 weeks if they became parasitemic or for 8 weeks if they did not become parasitemic. Parasitemic volunteers were treated with a standard course of chloroquine for chloroquine-sensitive *Pf* strains; for the chloroquine-resistant *Pf* strain (7G8), a standard course of mefloquine was given. Repeated challenges were performed on immunized volunteers to assess the duration of protection and to study the effects of secondary immunizations. The 3 *Pf* strains or clones used for challenges were NF54, the 3D7 clone of NF54, and the 7G8 clone of IMTM22/Brazil [31]. We consider NF54 and the 3D7 clone of NF54 to be heterologous to the 7G8 clone, for reasons that include the following: First, 3D7 was cloned from NF54, which was isolated from a Dutch person who lived near the Amsterdam airport. It is thought that the parasite came from West Africa. 7G8 was cloned from the IMTM-22 isolate from Brazil. Second, NF54 and 3D7 are sensitive to chloroquine, and 7G8 is resistant to chloroquine. Third, there are $\geqslant 6$ known variations in T cell epitopes on the *Pf* circumsporozoite protein ($n = 3$) and sporozoite surface protein 2 ($n = 3$) between the 3D7 and 7G8 sequences. A volunteer was considered to be protected if parasites were never detected in the bloodstream during follow-up.

Results

Safety and Tolerability and Reactions to Immunizations

In our experience with 165 separate mosquito immunizations in 13 volunteers, 12 of whom were challenged, the procedure

was well tolerated, without an unexpected adverse event. No volunteer withdrew from the study due to complications resulting from mosquito immunization or from the malaria challenge. Table 1 shows data on the cumulative number of exposures to immunizing mosquitoes, the total number of immunizing mosquitoes to which each volunteer was exposed, and the number of days from first to last exposure. During the feeding, volunteers usually reported some mild discomfort, but none requested cessation of exposure due to discomfort. After removal of the mosquito containers, a depressed ring corresponding to the container's circumference was visible and palpable on the volunteer's epidermis secondary to pressure contact between the container and skin. The epidermis enclosed by this ring usually had a mild erythematous hue studded by focal moderately erythematous papules. However, some persons were notably less reactive to the immunizations and displayed only a few mildly erythematous papules.

A mild generalized swelling of the skin within the confines of this ring developed in most volunteers, with the papules coalescing into a confluent erythematous plaque within minutes to an hour after the immunization. One volunteer, upon each immunization, developed focal wheals within the confines of the ring that then coalesced. In this subject, a 2-cm flare extended beyond the limits of the ring. The focal swelling was not associated with loss of function in the immunized arm and resolved within 24–72 h. No volunteer developed any symptom or sign suggestive of a systemic anaphylactic reaction.

Table 1. Summary of immunization and challenge studies at the Naval Medical Research Center and the Walter Reed Army Institute for Research, 1989–1999.

Volunteer	Cumulative no. before challenge: Immunizations	Cumulative no. before challenge: Immunizing bites	Day of challenge[a]	Weeks between last immunization and challenge	Protection[b]	Challenge with *Pf* strain
1	8	606	2	365	No	Homologous
3	9	1007	2	364	Yes	Homologous
4	8	1046	2	243	Yes	Homologous
	10	1310	2	316	Yes	Heterologous (7G8)
	12	1601	9	621	Yes	Homologous
	—	1601	42	847	Yes	Homologous
	—	1601	257	2357	No	Homologous
	13	1748	2	3080	Yes	Homologous
	16	2211	6	3733	Yes	Homologous
5	8	1001	2	243	Yes	Homologous
	10	1297	2	316	Yes	Heterologous (7G8)
	12	1585	8	621	Yes	Homologous
	—	1585	41	847	Yes	Homologous
	21	2927	6	3733	Yes	Homologous
9	9	1102[c,d]	—	—	—	—
10	10	1090[d]	3	250	Yes	Homologous
	—	1090	36	502	Yes	Homologous
	16	1872	7	3362	Yes	Homologous
11	10	1093	9	285	Yes	Homologous
	11	1214	23	511	Yes	Homologous
	15	1636[c]	—	—	—	—
12	11	1130	3	235	Yes	Homologous
	—	1130	36	487	No	Homologous
	12	1217[c]	—	—	—	—
15	10	1008	10	528	No	Homologous
	11	1212[c]	—	—	—	—
16	9	1127	2	550	Yes	Homologous
	17	2290	5	2826	Yes	Homologous
17	8	1163	2	551	Yes	Homologous
	11	1537[c]	—	—	—	—
18	8	1043	2	309	Yes	Homologous
	14	1911[c]	—	—	—	—
19	5	1050	2	113	Yes	Homologous
	6	1149[c]	—	—	—	—

NOTE. Radiation dose was 15 krad for all immunizations. *Pf, Plasmodium falciparum.*
[a] Day 0, first immunization.
[b] No patent parasitemia after the bites of 5 infected nonirradiated mosquitoes.
[c] Dropped out or relocated. No further challenges.
[d] Volunteers 9 and 10 were previously infected once with *Pf* 7G8 before any immunization, when serving as infectivity control subjects.

A tightly circumscribed erythematous papular rash characterized the immunization sites in some persons after 72 h and completely resolved in 7–10 days. Weeping, pustules, crusting, or other evidence of a secondary bacterial infection at the immunization sites did not occur. After resolution of the erythematous papular rash, the immunization sites of some volunteers had mildly hyperpigmented macules. However, within a few weeks, the immunization site in these persons was indistinguishable from adjoining skin.

The evening after some immunization sessions, a few volunteers reported a mild headache and/or malaise that completely resolved by the following morning. Volunteers did not report other constitutional symptoms and never modified their activities or lost work secondary to the reactions they experienced.

Protection

Protection against first challenge with homologous Pf *strain after primary immunization with >1000* Pf-*irradiated sporozoites.* Over the past 10 years at the NMRC/WRAIR, 11 white male volunteers, enrolled at ages 19–44 years (mean, 36), were challenged with infective bites from 5 mosquitoes infected with a homologous *Pf* strain after receiving >1000 immunizing bites from *Pf*-infected mosquitoes exposed to 15,000 rad of gamma radiation (table 1). In this primary immunization series, the volunteers received a mean of 9 immunizations, a mean of 123 immunizing bites per immunization, and 1001–1163 immunizing bites before their first challenge. Ten volunteers were first challenged 2–9 weeks after their last primary immunization, and all were protected. One volunteer (table 1, no. 15) was first challenged 10 weeks after his last primary immunization and was not protected. A 12th volunteer (table 1, no. 9) served as an infectivity control before completing 9 primary immunizations and was not challenged.

At the University of Maryland [20, 21], 3 volunteers (table 2, subjects 6–8) were challenged with infective bites from 5 mosquitoes infected with the CVD1 clone of *Pf* NF54 strain after receiving >1000 immunizing bites from *Pf*-infected mosquitoes exposed to 17,000–24,710 rad (mean, 20,820). In the primary immunization series, the volunteers received 19 immunizations, a mean of 86 immunizing bites per immunization, and 1563–1681 immunizing bites before their first challenge. These volunteers were first challenged 3 weeks after their last primary immunization, and all were protected.

On the basis of combined data from these institutions, all 13 volunteers challenged 2–9 weeks after receiving >1000 immunizing bites in a primary immunization series were protected. One volunteer (no. 15), challenged 10 weeks after receiving 1008 immunizing bites in his primary immunization series, was not protected. None of the earlier reports by Clyde and colleagues [2–5] or Rieckmann and colleagues [6–8] included volunteers who had received >1000 bites by infected irradiated mosquitoes before first challenge.

Reduced protection against first challenge with homologous Pf *strain after primary immunization with <1000* Pf-*irradiated sporozoites.* At both the NMRC/WRAIR and the University of Maryland, the level of protection appeared to be less for volunteers who received <1000 primary immunizing bites. At the NMRC/WRAIR, 1 volunteer (table 1, no. 1) was challenged 2 weeks after receiving 606 immunizing bites from mosquitoes irradiated at 15,000 rad and was not protected. In this case, the lack of protection was probably due to an insufficient number of immunizing bites.

At the University of Maryland, 2 volunteers (table 2, subjects 4 and 5) underwent a homologous challenge 5 weeks after receiving 625 and 715 immunizing bites from *Pf* NF54 strain–infected mosquitoes irradiated with 20,000–27,000 rad (mean, 23,610) and were not protected. The lack of protection in these 2 volunteers likely resulted from an insufficient number of immunizing bites but could have been due to overattenuation of the sporozoites by excessive radiation [32].

Table 2. Summary of immunizations and challenges with homologous *Plasmodium falciparum (Pf)* strain at the University of Maryland during the late 1980s and early 1990s [20, 21].

Volunteer	Radiation dose range (mean), krad	Cumulative no. before challenge: Immunizations	Cumulative no. before challenge: Immunizing bites	Weeks between last immunization and challenge	Day of challenge[a]	Protection[b]
4	20–27 (23.61)	7	625	5	129	No
5	20–27 (23.71)	11	715	5	312	No
6	17–25 (20.82)	19	1642	3	113	Yes
	—	20	1894	—	—	—
7	17–25 (20.82)	19	1563	3	113	Yes
	—	20	1771	—	—	—
8	17–25 (20.82)	19	1681	3	113	Yes
	—	20	1880	39	490	Yes

NOTE. Volunteers 4 and 5 were immunized and challenged with *Pf* NF54. Volunteers 6–8 were immunized and challenged with the *Pf* CVD1 clone of NF54.

[a] Day 0, first immunization.

[b] No patent parasitemia after the bites of 5 infected nonirradiated mosquitoes.

In the studies done by Clyde and colleagues [2–5], Rieckmann and colleagues [6–8], and McCarthy and Clyde [9] (table 3), all volunteers were first challenged after receiving <1000 immunizing bites from mosquitoes irradiated with a minimum of 12,000 or 15,000 rad. Five volunteers (EN, RP, GZ, LA, and DS) were challenged 2 weeks after receiving 379–954 primary immunizing bites before first challenge with a homologous strain. Three of the 5 were protected when challenged with 7–14 infective bites from nonirradiated mosquitoes, including the 2 with the most primary immunizing bites (440 and 954; LA and DS, respectively). Four additional volunteers were challenged ~2 weeks after receiving <200 primary immunizing bites and were not protected. These persons are not described in table 3. The lack of protection in these volunteers was probably the result of an insufficient number of immunizing bites.

When we combined the data from our studies, those at the University of Maryland, and the studies of Clyde et al. [2–5] and Rieckmann et al. [6–8], only 3 of the 8 volunteers challenged 2–5 weeks after receiving >200 and <1000 immunizing bites in

Table 3. Summary of immunization and challenge studies done in the 1970s, excluding persons who received <200 immunizing bites [2–9].

		Cumulative no. before challenge						
Volunteer	Radiation dose, krad	Immunizations	*Pf* immunizing bites	*Pv* immunizing bites	Weeks between last immunization and challenge	Day of challenge[a]	Protection[b]	Challenge with *Pf* strain unless *Pv* indicated
EN	12–15[c]	6	379	—	2	98	No	Homologous
RP	12–15[c]	6	379	—	2	98	No	Homologous
GZ	12–15[c]	6	379	—	2	98	Yes	Homologous
	15	11	1189	—	2	327	Yes	Homologous
	15	12	1309	—	1	413	Yes	Heterologous
	15	13	1441	—	2	435	No	*Pv*
	15	—	—	—	4	448	Yes	Heterologous
	15	—	—	—	5	459	Yes	Heterologous
DFC	15[c]	14	838	—	1	129	No	*Pv*
	15	—	—	—	2	139	No	Heterologous
	15	24	1806	—	1	206	Yes	Heterologous
	15	—	—	—	3	230	Yes	Heterologous
	15	34	—	539[d]	2	367	Yes	*Pv* homologous strain
	15	37	2206	—	12 (after *Pf* Imm)	427	No[e]	Heterologous
	15	—	—	—	12 (after *Pv* Imm)	443	Yes[f]	*Pv* heterologous strain
	15	—	—	—	26 (after *Pv* Imm)	536	No	*Pv* homologous strain
WK	17.5	3	—	728[f,g]	1	25	No[f]	*Pv* homologous strain
	17.5	7	—	1979 (1251[d])	1	123	Yes[d]	*Pv*
	17.5	—	—	—	23	276	No[d]	*Pv*
	17.5	—	—	—	27	307	Yes[d]	*Pv*
	17.5	—	—	—	40	398	Yes[f]	*Pv*
LA	12	6	440	—	2	84	Yes	Homologous
	12	—	—	—	16	182	No[h]	Homologous
DS	12	8	954	—	2	224	Yes	Homologous
	12	—	—	—	8	266	Yes	Homologous
	12	—	—	—	17	329	No	Heterologous
	12	—	—	—	25	385	No	Homologous
WD	12	7	987	—	8	322	Yes	Heterologous
	12	—	—	—	18	392	No	Homologous

NOTE. Imm, immunization; *Pf, Plasmodium falciparum; Pv, P. vivax.*

[a] Day 0, first immunization.

[b] No patent parasitemia after bites of 5–14 infected nonirradiated mosquitoes.

[c] For EN, RP, and GZ, 12 krad (mean, 14 krad) was the minimum irradiation for the first 4 immunizations, and 15 krad (mean, 17.5 krad) was the minimum irradiation in later immunizations. EN developed a breakthrough infection after immunization 3 with *Pf* sporozoites irradiated with a minimum of 12 krad. For DFC, 15 krad (mean, 17.5 krad) was the minimum irradiation for all immunizations. DFC had a complicated immunization and challenge schedule with 6 strains of *Pf* malaria (see [6] for details). DFC was immunized with bites of *Pv* Chesson sporozoites, and his heterologous *Pv* challenge was with *Pv* El Salvador. DFC had breakthrough infection of *Pv* malaria after immunization with *Pv* sporozoites, thought to be secondary to sporozoites escaping attenuation with 15 krad of radiation.

[d] Chesson strain.

[e] Volunteer challenged with bites of 90 infected nonirradiated mosquitoes.

[f] El Salvador strain.

[g] This volunteer was initially immunized by bites of 728 mosquitoes carrying *Pv* El Salvador sporozoites and was challenged with *Pv* El Salvador sporozoites. He was then boosted with additional bites of 1251 mosquitoes carrying *Pv* Chesson sporozoites, challenged 3 times with *Pv* Chesson sporozoites, and challenged with *Pv* El Salvador sporozoites.

[h] Volunteer was challenged with bites of 45 infected nonirradiated mosquitoes.

primary immunization series were protected ($P = .0028$, Fisher's exact test, 2-tailed, >1000 vs. <1000 immunizing bites). If the numbers are expanded to include subjects immunized with <200 infected irradiated mosquitoes, only 3 of 12 were protected.

Protection against repeated challenges (rechallenge) with homologous Pf *strain in volunteers immunized with irradiated* Pf *sporozoites.* At the NMRC/WRAIR, 6 volunteers (table 1, subjects 4, 5, 10–12, and 16) were rechallenged with homologous *Pf* strain sporozoites 1–5 times (13 additional rechallenges with the homologous strain). These rechallenges were done 2–257 weeks after a subject's last immunization. As shown in table 1, rechallenges occurred after the initial challenge or after a previous rechallenge and either with or without secondary immunization(s). Four volunteers (subjects 4, 5, 10, and 16) underwent 7 rechallenges 2–9 weeks after receiving a secondary immunization, and all were protected. One volunteer (no. 11) was rechallenged 23 weeks after receiving a secondary immunization and was protected. Two volunteers (10 and 12) were rechallenged 36 weeks after their last primary immunization: Subject 10 was protected, but subject 12 was not. Volunteers 5 and 4, respectively, were rechallenged 41 and 42 weeks after their last secondary immunization, and both were protected. Finally, volunteer 4 was rechallenged 257 weeks (almost 5 years) after a secondary immunization and was not protected. Of note, this volunteer then received a secondary immunization of 147 bites and was protected when rechallenged 2 weeks later.

At the University of Maryland, 1 volunteer (table 2, no. 8) was rechallenged 39 weeks after receiving a secondary immunization and was protected. From the studies by Clyde et al. [2–5], Rieckmann et al. [6–8], and McCarthy and Clyde [9] (table 3), fewer interpretable data are available on the longevity of homologous *Pf* protection. In their studies, most volunteers received *Pv* challenges or rechallenges (subjects GZ, DFC, and WK), received heterologous *Pf* challenges or rechallenges (subjects GZ, DFC, DS, and WD), were never rechallenged with a homologous *Pf* strain (subject WD), received <1000 primary immunization bites, or were unrealistically rechallenged by 45 infected nonirradiated mosquitoes (subject LA). Subject DS had a primary immunization of 954 bites and was protected when first challenged at 2 weeks and when rechallenged at 8 weeks with a homologous *Pf* strain. Volunteer GZ had a primary immunization of 379 bites and was protected when first challenged 2 weeks later with a homologous *Pf* strain. He then received an additional 810 boost immunization bites (total, 1189) and was protected when rechallenged 2 weeks later with a homologous *Pf* strain.

Combining the interpretable data from all these studies yielded 14 cases of protection in 15 homologous rechallenges done 2–42 weeks after the last primary immunization or the most recent secondary immunization. Of the 9 rechallenges conducted at weeks 2–9, all resulted in protection. Of the 6 rechallenges between weeks 23 and 42, 5 resulted in protection (table 4).

Protection against challenge with heterologous Pf *strains in volunteers immunized with* Pf-*irradiated sporozoites.* At the NMRC/WRAIR, 2 volunteers (table 1, subjects 4 and 5), after primary and secondary immunization with 1310 and 1297 *Pf* 3D7 strain bites, respectively, were protected when challenged 2 weeks later by the heterologous *Pf* 7G8 clone of IMTM/Brazil. In the studies by Clyde et al. [2–5] and Rieckmann et al. [6–8], 2 volunteers (table 3, subjects GZ and DFC), who received >1000 immunizing bites, were protected when challenged by a heterologous *Pf* strain on 5 occasions, excluding an unrealistic challenge with 90 nonirradiated infective mosquitoes. Three subjects (DFC, DS, and WD) received <1000 immunizing bites before challenge by a heterologous *Pf* strain, and 1 was protected and 2 were not on 3 occasions. Volunteer GZ underwent a heterologous *Pf* challenge 1 week after completing 12 immunizations (1309 bites) and underwent 2 sequential heterologous *Pf* rechal-

Table 4. Summary of all *Plasmodium falciparum (Pf)* challenges with 5–14 infected mosquitoes in volunteers immunized with radiation attenuated with *Pf* sporozoites.

No. of immunizing bites and challenges or rechallenges within 10 weeks or 23–42 weeks of last immunization	No. of volunteers protected/total no. challenged	No. of protected challenges/total no. of challenges (%)
>1000 Immunizing bites		
First challenged within 10 weeks of last immunization	13/14	13/14 (93)
Rechallenged within 10 weeks of last immunization	6/6	15/15 (100)
Rechallenged 23–42 weeks after last immunization	5/6	5/6 (83)
<1000 Immunizing bites		
First challenged within 10 weeks of last immunization	4/10	4/10 (40)
Rechallenged within 10 weeks of last immunization	1/1	1/1 (100)
Rechallenged >10 weeks after last immunization	0/3	0/4

NOTE. Data exclude volunteers immunized with <200 bites.

lenges 4 and 5 weeks after a secondary immunization of 132 bites (total, 1441) and was protected on all 3 occasions. Volunteer DFC underwent heterologous *Pf* rechallenges 1 and 3 weeks after completing several immunizations ($n = 1806$ bites) and was protected on both occasions. Volunteer DFC also underwent a heterologous *Pf* rechallenge with 90 infected nonirradiated mosquitoes 12 weeks after completing secondary immunizations (400 additional bites; total bites, 2206) and was not protected. Three volunteers (DFC, DS, and WD) received <1000 immunizing bites before challenge with a heterologous *Pf* strain on 3 occasions 2–17 weeks after their last immunization: Only WD, who received the greatest number of immunizing bites (987), was protected when challenged at 8 weeks.

In combined data from these studies, all 4 volunteers who received >1000 immunizing bites were protected against all 7 challenges or rechallenges from 5–10 mosquitoes infected with heterologous *Pf*. One volunteer who received >1000 immunizing bites was not protected against a challenge by 90 mosquitoes infected with heterologous *Pf*. Of the volunteers who received <1000 immunizing bites, only 1 challenge in 3 resulted in protection. Table 5 summarizes data on heterologous challenge after >1000 immunizing bites.

Protection against cross-species challenge. In the studies by Clyde et al. [2–5], Rieckmann et al. [6–8], and McCarthy and Clyde [9], 1 volunteer (table 3, subject GZ) who had received 1309 immunizing *Pf* bites and had been protected against 3 *Pf* challenges was not protected against a *Pv* challenge 2 weeks after his last *Pf* immunization. This is the only pure cross-species challenge after >1000 immunizing bites. A second volunteer (DFC) underwent sequential *Pv* and heterologous *Pf* challenges 1 and 2 weeks after completing a primary immunization of 838 *Pf* bites and was not protected on either occasion. He then received secondary immunizations totaling 968 homologous *Pf* bites and underwent 2 sequential heterologous *Pf* challenges at 1 and 3 weeks and was protected on both occasions. DFC then received 539 immunizing *Pv* (El Salvador strain) bites and underwent a homologous *Pv* challenge 2 weeks later and was protected. He then received secondary immunizations with 400 homologous *Pf* bites and underwent a heterologous *Pf* challenge 12 weeks later and was protected. After that, he underwent a heterologous *Pv* challenge 12 weeks after his immunization with *Pv* sporozoites and was protected. He had a homologous *Pv* challenge 26 weeks after his immunization with *Pv* sporozoites and was not protected. The analysis of cross-species protection in DFC is confounded by his mixed immunization with *Pf* and *Pv* sporozoites. Table 5 summarizes data on cross-species challenge.

Protection against challenge with Pv *in volunteers immunized with* Pv*-irradiated sporozoites.* Rieckmann et al. [7] reported that 3 volunteers were not protected against homologous *Pv* challenge after receiving <200 immunizing bites. One volunteer (table 3, subject WK) was not protected on his first homologous challenge 1 week after 728 immunizing bites with the *Pv* El Salvador strain [9]. After an additional 1251 immunizing bites from the Chesson strain (cumulative bites from both strains, 1979), he underwent 3 successive Chesson strain challenges 1, 23, and 27 weeks after his last immunization. He was protected at 1 and 27 weeks but not at an intervening challenge at 23 weeks. At 40 weeks, he was protected against an El Salvador strain challenge. Volunteer DFC, whose immunizations were described in the prior paragraph, was immunized with both *Pf*- and *Pv*-irradiated sporozoites. He was protected after a homologous *Pv* challenge 2 weeks after and a heterologous *Pv* challenge 12 weeks after receiving 539 immunizing bites from irradiated *Pv*-infected mosquitoes but was not protected after a homologous *Pv* challenge at 26 weeks after a previous immunization. Again, interpretation of these results is clouded by the 2 species immunization regimen. Table 5 summarizes data on *Pv* immunization and *Pv* challenges.

Validity of challenges. At the NMRC/WRAIR, 25 infectivity control volunteers (mean, 3 control volunteers per challenge) were used for the challenges, and all control volunteers became parasitemic. Similarly, at the University of Maryland, where there were 11 infectivity control volunteers, all became parasitemic, with the exception of 1 volunteer who had limited symptoms on days 14 and 15 but had negative thick blood smears between days 5 and 30. However, his blood cultures were positive for *Pf* on days 21 and 30 [15]. In the reports by Clyde et al. [2–5], Rieckmann et al. [6–8], and McCarthy and Clyde [9], 28 paired infectivity control volunteers were noted, and all became parasitemic.

Breakthrough blood-stage infections. In the studies at the NMRC/WRAIR and in recent University of Maryland studies, all immunizing mosquitoes received 15,000 rad (NMRC/WRAIR) or more (University of Maryland), and no breakthrough blood-stage infections occurred. In a study by Clyde [5], 4 of 7 volunteers immunized by sporozoites irradiated with 12,000 rad developed breakthrough infections. In a study by Rieckmann [8],

Table 5. Summary of *Plasmodium falciparum (Pf)* and *P. vivax (Pv)* challenges in volunteers who received >1000 immunizing bites from *Pf* infected mosquitoes within 1 year of their last primary or secondary immunization.

Immunization and challenge	No. of volunteers protected/total no. challenged	No. of protected challenges/total no. of challenges (%)
Pf immunization, *Pf* homologous challenge (5–14 mosquitoes)	14/15	26/28 (92)
Pf immunization, *Pf* heterologous challenge (5–10 mosquitoes)	4/4	7/7 (100)
Pf or *Pv* immunization, *Pv* challenge (5–14 mosquitoes)	0/2[a]	1/4 (25)[b]
Pv immunization, *Pv* challenge (5–12 mosquitoes)	1/1	3/4 (75)

[a] Subjects GZ and DFC from table 3.
[b] Only subject GZ had a pure *Pv* challenge after immunization with >1000 immunizing bites from irradiated *Pf*-infected mosquitoes without *Pv* immunizations. Subject DFC had mixed immunization with both irradiated *Pf*- and *Pv*-infected mosquitoes before challenge with nonirradiated *Pv* mosquitoes.

2 of 11 volunteers immunized by sporozoites irradiated with 12,000 rad developed breakthrough infections. In addition, subject DFC had a breakthrough infection after immunization that was thought to be secondary to sporozoites escaping attenuation with 15,000 rad [2].

In combined data from these studies, 6 (all exposed to *Pf*) of 18 volunteers developed malaria after exposure to infected mosquitoes irradiated with 12,000 rad, likely due to insufficient irradiation of the sporozoites. In the single breakthrough infection after mosquito exposure to 15,000 rad, there was a concern regarding the number of rads actually delivered.

Discussion

Data from the NMRC/WRAIR studies, when combined with the results of other studies, clearly demonstrate that humans receiving >1000 bites from *Pf*-infected mosquitoes irradiated with 15,000 rad are protected against primary *Pf* challenge for at least 9 weeks after last exposure and against rechallenges for at least 23–42 weeks. They also demonstrate that protection is not specific to the *Pf* strain used for immunization. No data were generated in our studies regarding cross-species protection against *Pv* after immunization with irradiated *Pf* sporozoites. Of importance, exposure to thousands of irradiated *Pf*-infected mosquitoes was safe and generally well tolerated.

Table 4 summarizes the results of challenges, divided by the number of immunizing bites (>1000 vs. <1000 bites) and the interval between immunization and challenge (⩽10 vs. >10 weeks). Overall, 33 of 35 challenges within 42 weeks after >1000 immunizing bites led to protection, whereas only 5 of 15 challenges after >378 and <1000 immunizing bites led to protection ($P < .0001$, Fisher's exact test, 2-tailed). This indicates that ~1000 bites provide essentially complete protection against sporozoite challenge and that fewer bites may or may not be associated with protection.

The number of immunization sessions did not seem to affect protection. In our studies (table 1), volunteers receiving >1000 immunizing bites were protected regardless of whether primary immunizations took place in 5 sessions (1 volunteer), 8 sessions (4 volunteers), 9 sessions (2 volunteers), 10 sessions (2 volunteers), or 11 sessions (1 volunteer). Our single failure to protect after first challenge after >1000 immunizing bites occurred after 10 immunization sessions. Three volunteers shown in table 2 were also protected against first challenge after receiving >1000 immunizing bites—each of these volunteers was immunized in 19 sessions.

Our 11 volunteers who received >1000 immunizing bites were first challenged at 2–10 weeks after the last immunization. The single subject first challenged at 10 weeks was not protected, whereas intervals of 2 weeks (7 volunteers), 3 weeks (2 volunteers), and 9 weeks (1 volunteer) before first challenge were all associated with protection. Repeated challenges after secondary immunization showed that protection is long lasting. In our studies (5 volunteers), and the recent University of Maryland studies (1 volunteer), 5 of 6 volunteers challenged 23–42 weeks after the most recent secondary immunization were protected. Protection was achieved at 23, 36, 39, 41, and 42 weeks (1 volunteer for each period) after secondary immunization. One failure occurred 36 weeks after the last immunization. For longer intervals, we have only a single data point: 1 volunteer challenged 257 weeks (5 years) after the last immunization who was not protected. This volunteer was treated, was given a booster immunization with 147 immunizing bites, and was protected 2 weeks after receiving this boost.

The number of mosquito bites used for challenge may also affect protection. Under natural conditions of exposure, persons are rarely bitten by >1 infected mosquito per night. We used 5 experimental bites for all challenges. Only 50% of humans bitten by 1 or 2 *Pf*-infected *A. stephensi* mosquitoes developed parasitemia [29], whereas nearly 100% of humans bitten by 5 *Pf*-infected mosquitoes developed parasitemia [29, 30, 33]. In an early study [4], 1 volunteer (table 3, subject DFC) was challenged with 90 infected nonirradiated mosquitoes and was not protected, despite 2206 immunizing bites and a 12-week interval between his last immunization and challenge (table 3). This is an unrealistic challenge that we believe has little relevance to this vaccine model. Nonetheless, it suggests that dramatically increasing the challenge dose may overcome the irradiated sporozoite vaccine–induced protection.

All 7 heterologous challenges, in 4 volunteers who were challenged 1–5 weeks after their last immunization, resulted in protection (table 5). These data indicate that the irradiated sporozoite vaccine induces strain-transcendent protection, but the number of strains that have been tested is small, and exposure in the field will be with many different strains.

Interpretation of *Pv* data was confounded because volunteers received mixed immunizations with both *Pf* and *Pv* sporozoites before challenge. One volunteer received >1000 immunizing *Pv* bites in the absence of *Pf* immunization and was protected in 3 of 4 *Pv* challenges (table 3, subject WK). With regard to cross-species challenges, there is one clear instance of a volunteer (subject GZ) immunized only by >1000 bites of *Pf*-infected irradiated mosquitoes (1309 bites) and challenged with *Pv* (2 weeks after the last immunization) who was not protected against *Pv* challenge (table 3). In a second instance, volunteer DFC was challenged with *Pv* after exposure to 838 *Pf*-infected mosquitoes and was not protected (table 3). It will be important to actually determine whether immunization with *Pf*- or *Pv*-irradiated sporozoite does or does not consistently protect against cross-species challenge.

Protection lasted for at least 42 weeks in these studies. However, it is not known whether protection requires the ongoing presence of irradiated parasites in infected hepatocytes. A study [34] in mice treated with primaquine to eliminate hepatic-stage parasites suggested that persistence of irradiated sporozoites in hepatocytes is necessary to maintain protective im-

munity. If the protection resulting from the irradiated sporozoite vaccine in humans depends on the ongoing presence of attenuated hepatic-stage parasites and does not induce immunologic memory with the potential for an anamnestic response after challenge, its usefulness as a model for subunit malaria vaccine development may be limited.

We used mosquitoes exposed to 15,000 rad. Exposure of volunteers to mosquitoes that received 12,000 rad resulted in protection in some cases but was also associated with breakthrough infections, presumably due to insufficient attenuation [2, 8]. Exposure of volunteers to mosquitoes receiving $>20,000$ rad may fail to protect due to overattenuation of the sporozoites. In cultured human hepatocytes, there is an inverse association between the number of sporozoites capable of penetration, limited development within the hepatocytes, and radiation dose [32]. Most investigators believe that 15,000–20,000 rad is optimal [2–5, 13–15].

The irradiated sporozoite vaccine is an excellent model for malaria vaccine development, because irradiated sporozoites enter hepatocytes and only partially develop within these cells. Thus, volunteers immunized with irradiated sporozoites do not develop clinical symptoms of malaria. They only develop immune responses against antigens expressed by irradiated sporozoites and against antigens expressed when the irradiated sporozoites partially develop within hepatocytes. They do not make immune responses against the majority of erythrocytic-stage antigens, since they are not expressed by irradiated sporozoites in liver cells. There is no progression to the sexual stage and hence no transmission of malaria. In contrast to serum and cells acquired from nonimmune persons with naturally acquired malaria, serum and cells from irradiated sporozoite–immunized volunteers can be used as specific probes for identifying protective parasite antigens expressed at the sporozoite and liver stages of the parasite life cycle.

The irradiated sporozoite vaccine provides critical data for defining mechanisms of protective immunity, defining antigenic targets of protective immunity, developing subunit malaria vaccines, developing assays that predict protective immunity, and validating reagents used to assess immune responses in clinical trials of experimental subunit vaccines. Of greatest importance, immunization of volunteers with irradiation-attenuated sporozoites demonstrates that it is feasible to develop a highly protective malaria vaccine and provides the foundation for work on developing a subunit preerythrocytic-stage malaria vaccine [35].

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